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# Ribosome engineering of *Myxococcus xanthus* for enhancing the heterologous production of epothilones

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## Abstract

**Background** Ribosome engineering is a semi-empirical technique used to select antibiotic-resistant mutants that exhibit altered secondary metabolism. This method has been demonstrated to effectively select mutants with enhanced synthesis of natural products in many bacterial species, including actinomycetes. Myxobacteria are recognized as fascinating producers of natural active products. However, it remains uncertain whether this technique is similarly effective in myxobacteria, especially for the heterologous production of epothilones in *Myxococcus xanthus*.

**Results** Antibiotics that target the ribosome and RNA polymerase (RNAP) were evaluated for ribosome engineering of the epothilone-producing strain *M. xanthus* ZE9. The production of epothilone was dramatically altered in different resistant mutants. We screened the mutants resistant to neomycin and rifampicin and found that the yield of epothilones in the resistant mutant ZE9N-R22 was improved by sixfold compared to that of ZE9. Our findings indicate that the improved growth of the mutants, the upregulation of epothilone biosynthetic genes, and specific mutations identified through genome re-sequencing may collectively contribute to the yield improvement. Ultimately, the total titer of epothilones achieved in a 10 L bioreactor reached 93.4 mg/L.

**Conclusions** Ribosome engineering is an efficient approach to obtain *M. xanthus* strains with enhanced production of epothilones through various interference mechanisms. Here, we discuss the potential mechanisms of the semi-empirical method.

**Keywords** Ribosome engineering, *Myxococcus xanthus*, Secondary metabolism, Production of epothilones, Metabolic interference

## Background

Ribosome engineering (RE) is a semi-empirical method employed to select mutants resistant to ribosome- or RNAP-associated antibiotics, and the mutations in ribosomal proteins or RNAP may stimulate the synthesis of secondary metabolites [1–3]. Hosaka et al. reported that 43% of the tested non-producing *Streptomyces* strains commenced antibacterial production upon acquisition of spontaneous mutations that conferred resistance to rifamycin (Rif) or streptomycin (Str) [1]. Mutations in the *rpsL* gene encoding the ribosomal protein S12 [4, 5] or the *rsmG* gene encoding 16S rRNA methyltransferase [6], were identified in the mutant strains resistant to Str, whereas mutants obtained by selection with Rif typically harbored mutations in the RNAP subunit (specifically in the r*poB* gene) [7]. ese

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RE mutations result in the structural and functional alterations in the ribosome or RNAP, thereby enhancing the production of natural active products (NAPs). Over the past two decades, many other ribosome-associated antibiotics, such as paromomycin (Par), gentamicin (Gen), and neomycin (Neo), have been successfully applied to select mutants with elevated production of NAPs, predominantly in *Streptomyces* genus [2, 3, 8, 9].

e combination of various drug-resistant mutations can further augment the bacterial productivity. For example, the introduction of octuple drug-resistant mutations in *Streptomyces coelicolor* resulted in a 180-fold increase in actinorhodin production [10]. Although RE has been extensively applied in *Actinomyces* to improve NAP production or to activate novel secondary metabolites, it has not, to our knowledge, been described in myxobacteria.

Myxobacteria, initially recognized for their fascinating social behaviors, are characterized by their abundant NAPs and are considered important drug-producing microorganisms, following actinomycetes and fungi [11, 12]. Epothilones are famous antitumor compounds isolated from some *Sorangium cellulosum* strains, from which two kinds of anticancer drugs have been developed and approved for the treatment of advanced breast cancer [13, 14]. Owing to the challenges associated with the low yield and the di culties in the genetic manipulation of *S. cellulosum*, the biosynthetic gene cluster (BGC) of epothilone has been expressed in various heterologous hosts; however, the yields obtained remain inferior to those in native producers [15]. *Myxococcus xanthus*, as a fermentation-friendly and genetically amenable host that is phylogenetically related to *S. cellulosum*, presented the highest initial yields of epothilones and exhibits considerable potential for application [15].

In previous research, we successfully expressed the epothilone BGC in *M. xanthus* DZ2 and obtained a high-yielding strain designated *M. xanthus* ZE9, in which the epothilone BGC was integrated into the DZ2 genome [16]. e production of epothilone in ZE9 was optimized at the transcriptional level through various strategies, including the modification of promoters [17], transcriptional regulators [18], and the application of CRISPR-dCas9-mediated transcriptional activation [19, 20]. In this study, we performed the RE in *M. xanthus* strain ZE9, and investigated its capacity to select mutants with stimulated production of secondary metabolites in *M. xanthus*.

## Materials and methods

### Strains and culture conditions

e strains used in this study are listed in Table S1. *M. xanthus* ZE9 and mutant strains were cultivated at 30 °C in CYE medium [5 g/L yeast extract, 10 g/L casitone, 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), and 4 mM $MgSO_4$, pH 7.4. For solid medium, 15 g/L agar was added]. CMO medium [CYE medium added with 7 mL/L of methyl oleate and 2% XAD-16 resin (Merck, Germany)] was used for fermentation. e medium was supplemented with the following antibiotics if required: apramycin [Apra, 30 μg/mL]; rifampicin [Rif, 2 μg/mL]; neomycin [Neo, 150 μg/mL]; paromomycin [Par, 200 μg/mL]; tetracycline [Tet, 10 μg/mL].

### Determination of MICs for antibiotics against *M. xanthus* ZE9

e minimum inhibitor concentrations (MICs) for di erent antibiotics were determined using microdilution in 96-well microplates. e antibiotics with a concentration of 256, 128, 64, 32, 16, 8, 4, 2, and 1 μg/mL were added to the microplate containing 150 μL of test microorganisms, which were diluted from the bacterial suspension cultured overnight ($OD_{600}$ is about 1) by 200 times. e microplates were incubated at 30 °C for 36 h, and the minimum antibiotic concentrations at which no visible growth was observed were considered as the MIC values.

### Ribosome engineering of *M. xanthus* ZE9

ZE9 was cultured overnight in 50 mL of CYE liquid medium. en, 1 OD of bacteria was mixed with soft agar and spread on CYE plates containing antibiotics (Rif, 2 μg/mL; Neo, 150 μg/mL; Par, 200 μg/mL; Tet, 10 μg/mL) and cultured at 30 °C. e drug-resistant colonies appeared after 6–7 days were transferred to new plates containing the same antibiotics and regrown to reconfirm resistance.

For combined selection with multiple antibiotics, the ZE9-N18 was incubated using the same method, and the mutant strains resistant to two antibiotics were selected on plates containing Neo plus Rif or plates containing Neo plus Par. e ZE9N-R14, ZE9N-R22, ZE9N-R25, ZE9N-R27, and ZE9N-R28 strains were cultured similarly and mixed together before being screened on plates containing Neo, Rif, and Tet.

### Genetic characteristics of mutant strains

Chromosomal DNAs of the *M. xanthus* mutants were extracted and purified with Bacteria Genomic DNA kit (TIANGEN, China). e potentially mutated genes, *rpoB* and *rpsL*, were amplified from Rif-resistant and Par-resistant mutants, respectively, and then sequenced at Tsingke Biotech Co., Ltd. (Beijing, China). Primers used are listed in Table S2.

e ZE9 genome was sequenced with PacBio Sequel II platform at Frasergen Genetic Information Co. Ltd.

(Wuhan, China). e genome was assembled using Microbial Assembly (smrtlink8) and annotated with Glimmer (v3.02) [21], tRNAscan-SE (v2.0) [22], and RNAmmer (v1.2) [23]. Genome re-sequencing was conducted with Illumina NovaSeq X Plus platform at Novogene Technology Co. Ltd. (Beijing, China). Sequence data was mapped with the ZE9 reference genome using BWA (parameters: mem -t 4 -k 32 -M) [24]. e SNPs and InDels were detected and annotated with GATK (parameters: HaplotypeCaller-pair-hmm-gap-continuation-penalty 10-genotyping-mode DISCOVERY-stand-call-conf 30) [25] and ANNOVAR [26]. e sequencing data was uploaded into the NCBI database as BioProject PRJNA1184456.

### Detection of the epothilone production in asks

e ZE9 and resistant mutants were cultured in 50 mL of CYE medium overnight and then inoculated into 50 mL of CMO medium at a ratio of 1:50. After 7 days of incubation, the resin was collected into the 15 mL centrifuge tubes in which 8 mL of methanol was added to extract the epothilones overnight. e leaching liquor was filtered with 0.22 μm filter and analyzed by high-performance liquid chromatography (HPLC) (SHIMADZU, Japan) as we previously described [17]. Briefly, 20 μL of filtered sample was used for the chromatographic separation with a C18 column (4.6 mm × 250 mm, 4.60 μm, ermo Scientific) at a flow rate of 1.0 mL/min, with mobile phase of 40% of $H_2O$ and 60% of methanol. According to the standard curve of epothilones, the yields of epothilones were quantified according to the formula $Y = (A \times 1.1 \times 10^{\wedge -7} - 3 \times 10^{\wedge -5}) \times 160$ (Y represents the yield of epothilone, and A presents the peak area of epothilone in the UV chromatogram).

### LC–MS analysis of secondary metabolites

e LC–MS analysis was performed on a UHPLC system ( ermo Scientific Vanquish, USA) coupled with a Q Exactive HFX Hybrid Quadrupole-Orbitrap mass spectrometer (Q Exactive HFX, ermo Scientific, USA) equipped with an ESI source. e chromatographic separation was done using a C18 column (4.6 mm × 250 mm, 4.60 μm, ermo Scientific, USA).

e elution solvent system was water and methanol, with a linear gradient from 5 to 100% methanol in water in 0–30 min, at a constant flow rate of 0.8 mL/min. ESI source parameters were used as follows: heater temperature 425 °C, sheath gas flow rate 50 arb, Aux gas flow rate 13 arb, Spray voltage ± 3500 V, and normalized collision energy 20, 40, 60 eV.

### Transcriptional analysis of epothilone genes

e transcription of epothilone genes were determined by real-time quantitative PCR (RT-qPCR) as we previously reported [17]. Briefly, cells were collected at 48 h during fermentation, followed with RNA extraction with BIOZOL kits (Total RNA Extraction Reagent, BioFlux, China). e total RNA was reverse-transcribed into cDNA with the PrimeScript™ Regent Kit with DNase (Takara, Japan) for the RT-qPCR analysis on LightCycler® 480 (Roche, Switzerland) with ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). e *gapA* gene (glyceraldehyde-3-phosphate dehydrogenase gene, *MXAN_2815*) was chosen as the reference gene for normalization. All the primers used are listed in Table S2.

### Detection of intracellular protein concentration

e strains were cultured in 50 mL CYE medium overnight and then inoculated into 50 mL CMO medium with initial 0.04 OD. 2 mL of suspension was harvested at 24 h and 48 h of incubation, washed with cold PBS bu er ($KH_2PO_4$ 0.27 g/L, $Na_2HPO4$ 1.42 g/L, NaCl 8 g/L, KCl 0.2 g/L, pH 7.4) three times and then disrupted with ultrasonic processor (5 s of sonication with 10-s interval was performed 4 times). After centrifugation of 12,000 rpm, 25 min at 4 °C, 2 μL of the protein supernatant was used for automated detection with NanoDrop2000C ( ermo Scientific, USA) in the protein-A280 mode.

### Fermentation of ZE9N-R22 in bioreactors

e ZE9N-R22 was incubated in CYE medium containing Neo (64 μg/ml) and Rif (2 μg/ml) overnight at 30 °C, and then inoculated into 3 L fermenter (BioFlo 310, Eppendorf NBS, Germany) with 2 L of CMO medium. e fermentation was performed at 30 °C for 7 days with dissolved oxygen (DO) controlled at 50%, while pH not controlled. For fed-batch fermentation in 10 L bioreactor (BioFlo 310, Eppendorf NBS, Germany) containing 5 L of CMO medium, nitrogen nutrition (casitone 150 g/L and yeast extract 75 g/L) and methyl oleate were added at feed rated of 140 ml /L and 6 ml/L, respectively. e feeds were started after 4 days of incubation, and delivered 5 times as a single shot every 48 h. Cultivation pH was maintained at 7.4 by automated addition of 2.5 M $H_2SO_4$, and the DO was controlled at 50%. e cells were allowed to grow for 18 days before being harvest together with resin, followed with extraction with 800 mL of methanol. e epothilone products were detected as mentioned above.

## Results

### Screening of antibiotics applicable for the RE of *M. xanthus* ZE9

Seven antibiotics commonly used in RE were evaluated for their MICs against *M. xanthus* ZE9 (Table 1). Mutations in the *rpsL* gene [5] or the *rsmG* gene [6] have been documented in streptomycin-resistant mutants, with analogous mutations in *rpsL* reported in mutants selected with Par [27]. In Rif-resistant mutants, mutations are localized within the *rpoB* gene [7]. Mutations in the ribosome influence the protein translation process, while mutations in *rpoB* may result in transcriptional-level regulation. e mutational mechanisms associated with Tet, Neo, Gen, and spectinomycin (Spe) remain to be elucidated.

As shown in Table 1, ZE9 was tolerant to Str, Gen, and Spe, with MIC values exceeding 256 μg/mL. e remaining four antibiotics, of which the Rif exhibited the strongest inhibitory activity, were used for subsequent experiments.

### Disruption of epothilone production in single antibiotic-resistant mutants

Resistant mutants were selected against Par, Rif, Neo, or Tet at concentrations higher than their MICs. Variations in mutant rate were observed during selection with individual antibiotic (Table 1). Ultimately, we obtained 19 Par-resistant mutants, 19 Rif-resistant mutants, 37 Neo-resistant mutants, and 8 Tet-resistant mutants. Notable phenotypic alterations were observed in certain mutant strains, including a change in colony color from bright yellow to bu , gray, or orange (Fig. 1a), which may be associated with disruptions in the synthesis of endogenous pigments.

All resistant mutant strains were fermented and subsequently evaluated for epothilone production. Among the Par-resistant mutants, 60% exhibited increased epothilone yields, while 25% demonstrated decreased yields, with a maximum variation of 70% (Fig. 1b). Similarly, approximately 50% of the Rif-resistant mutants produced less epothilones compared to ZE9, with the lowest yield of ZE9-R12 being only 23% of that of ZE9 (Fig. 1b).

Improved production of epothilones was observed in 22% of the Rif-resistant mutants, with a maximum increase of 40%. erefore, a significant proportion of the mutations selected by Rif and Par resulted in detrimental e ects on epothilone biosynthesis. In relation to the Neo-resistant mutants, 27% of the mutants exhibited reduced epothilone production, while a greater proportion (approximately 40%) presented increased titers (Fig. 1c). Among these mutants, ZE9-N29 showed the lowest epothilone yield, which was 91% lower than that in ZE9. Conversely, the yield of ZE9-N18 was increased by 400%, reaching 34.7 mg/L. Although only 8 Tet-resistant mutants were identified, 4 of these mutants were found to be more productive strains (Fig. 1c), suggesting that mutations selected by Tet may have a more positive e ect on epothilone synthesis, thereby generating more high-yielding mutants. is study demonstrated, for the first time, the di erences observed in mutants of *M. xanthus* ZE9 selected with di erent antibiotics, and their influences on epothilone production. ese findings undoubtedly serve as a reference for the future application of RE technique in myxobacteria.

### Selection of multiple antibiotics for mutants exhibiting improved epothilone production

With RE, disparate mutations may be selected by di erent antibiotics. Consequently, the desired mutations that stimulate the synthesis of target products can be integrated by successive selection through multiple antibiotics. To further screen high-yielding mutants, the Neo-resistant mutant ZE9-N18, which exhibited the highest epothilone yield, was chosen for sequential selection with Rif. Although selection with Par gave 60% of mutants with increased production, Par was un-adopted in combined

**Table 1** Antibiotics used for ribosome engineering of *M. xanthus* ZE9

| Antibiotics | Targets | Mutant gene | MIC (μg/mL) | Screening concentration (μg/mL) | Mutant rate |
|---|---|---|---|---|---|
| Streptomycin | 30S | *rpsL* or *rsmG* | > 256 | – | – |
| Gentamicin | 30S | *rplF* | > 256 | – | – |
| Spectinomycin | 30S | – | > 256 | – | – |
| Paromomycin | 30S | *rpsL* | 64 | 200 | $6 \times 10^{-7}$ |
| Rifampicin | β subunit of RNA polymerase | *rpoB* | 1 | 2 | $3 \times 10^{-8}$ |
| Neomycin | 30S | – | 64 | 150 | $1 \times 10^{-8}$ |
| Tetracycline | 30S | – | 8 | 10 | $1 \times 10^{-7}$ |

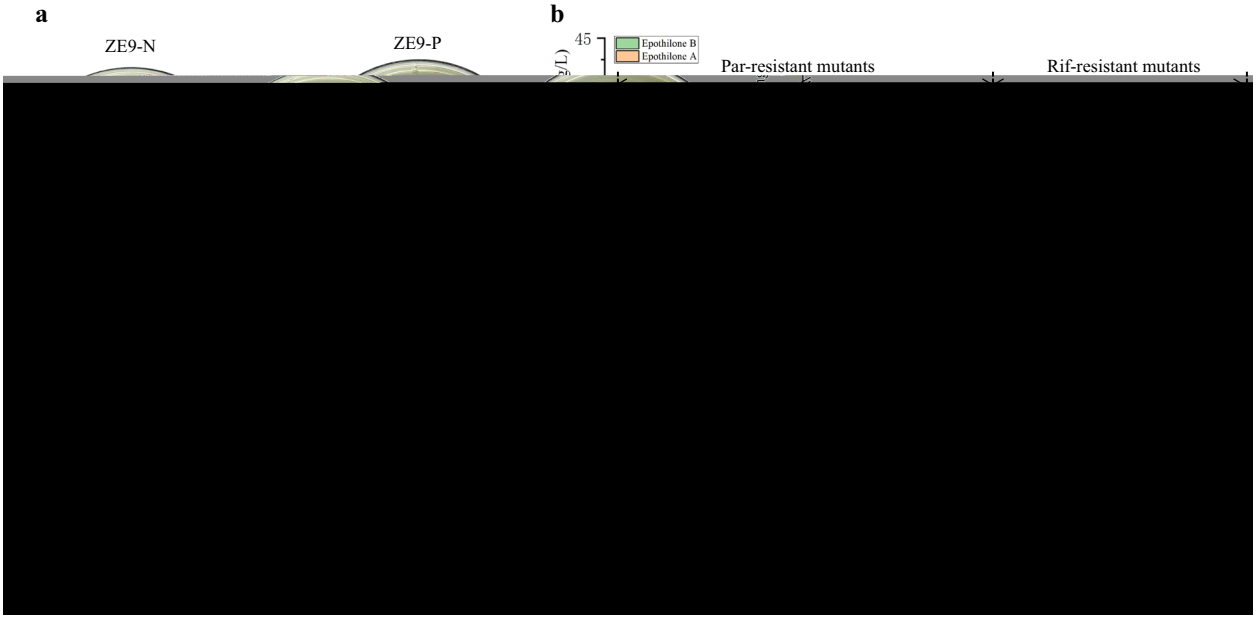


**Fig. 1** Colony growth and epothilone yields of mutant strains selected by single antibiotic. **a** Growth of mutant colonies. ZE9-N, mutants resistant to neomycin; ZE9-P, mutants resistant to paromomycin; ZE9-R, mutants resistant to rifampicin; ZE9-T, mutants resistant to tetracycline. **b** Detection of epothilone yields in ZE9-P and ZE9-R mutants. The error bars represent the standard deviation of three independent experiments. **c** Detection of epothilone yields in ZE9-N and ZE9-T mutants. The error bars represent the standard deviation of three independent experiments

selection due to the resistance of the ZE9-N18 strain to Par (MIC > 256 μg/mL). 62 double-resistant strains designated ZE9N-Rs were obtained; these strains displayed similar growth characteristics with minor color variations (Fig. 2a) but di erent epothilone productivities (Fig. 2b). In comparison to ZE9-N18, most ZE9N-R strains (73%) presented reduced epothilone yields, which was consistent with the observed e ect of Rif on ZE9. Among the five mutants with enhanced epothilone production (marked with red stars in Fig. 2b), the highest yield was improved by 62%, rising from 33.7 mg/L in ZE9-N18 to 54.8 mg/L in ZE9N-R22.

e five high-yielding dual-resistant strains, including ZE9N-R14, ZE9N-R22, ZE9N-R25, ZE9N-R27, and ZE9N-R28, were found to produce epothilones without any significant di erence (t-test, $p > 0.05$). e optimal strain for subsequent screening aimed at achieving mutants with higher yield of epothilones remains indeterminate. ese five strains were consequently mixed together for the selection of tri-resistant mutants with Tet. irteen mutants, named as ZE9NR-T strains, were isolated and further analyzed. As shown in Fig. 3, the mutants presented di erent colors, with the bright yellow stains demonstrating higher yields of epothilones compared to the o -white ones. However, the acquisition of the third resistance to Tet did not further promote the synthesis of epothilones among the tested mutants.

### Genotype identi□cation of high-yielding mutants

Mutations in ribosomes or RNAP selected by RE may a ect the global transcription and translation activities, consequently influencing the synthesis of natural products in mutant strains. is is considered the primary mechanism of RE [28]. erefore, we investigated how the mutations surviving antibiotic selection influence epothilone production in high-yield strains, in terms of genotype, growth, transcription, and product synthesis.

Among the four antibiotics used in this study, Rif and Par have been reported to select mutations in *rpoB* and *rpsL*, respectively. e target genes associated with Rif-resistant and Par-resistant mutants were amplified and sequenced. A total of three distinct single-base mutations in the *rpoB* gene were identified in 17 out of 19 Rif-resistant mutants (Table S1), including H548Y (11 strains), D538V (2 strains) and V152F (4 strains). Notably, the production of epothilones was una ected in the mutants harboring the D538V mutation (ZE9-R1 and ZE9-R2), whereas it was significantly impacted in the mutants containing the V152F or H548Y mutation (Fig. 1b). Among the strains carrying the H548Y mutation, variations in epothilone yields were observed, suggesting that the Rif-associated mutation in *rpoB* may not the primary determinant of epothilone yield variability. As to the Par-resistant mutants, decreased epothilone yields were observed in all 13 strains

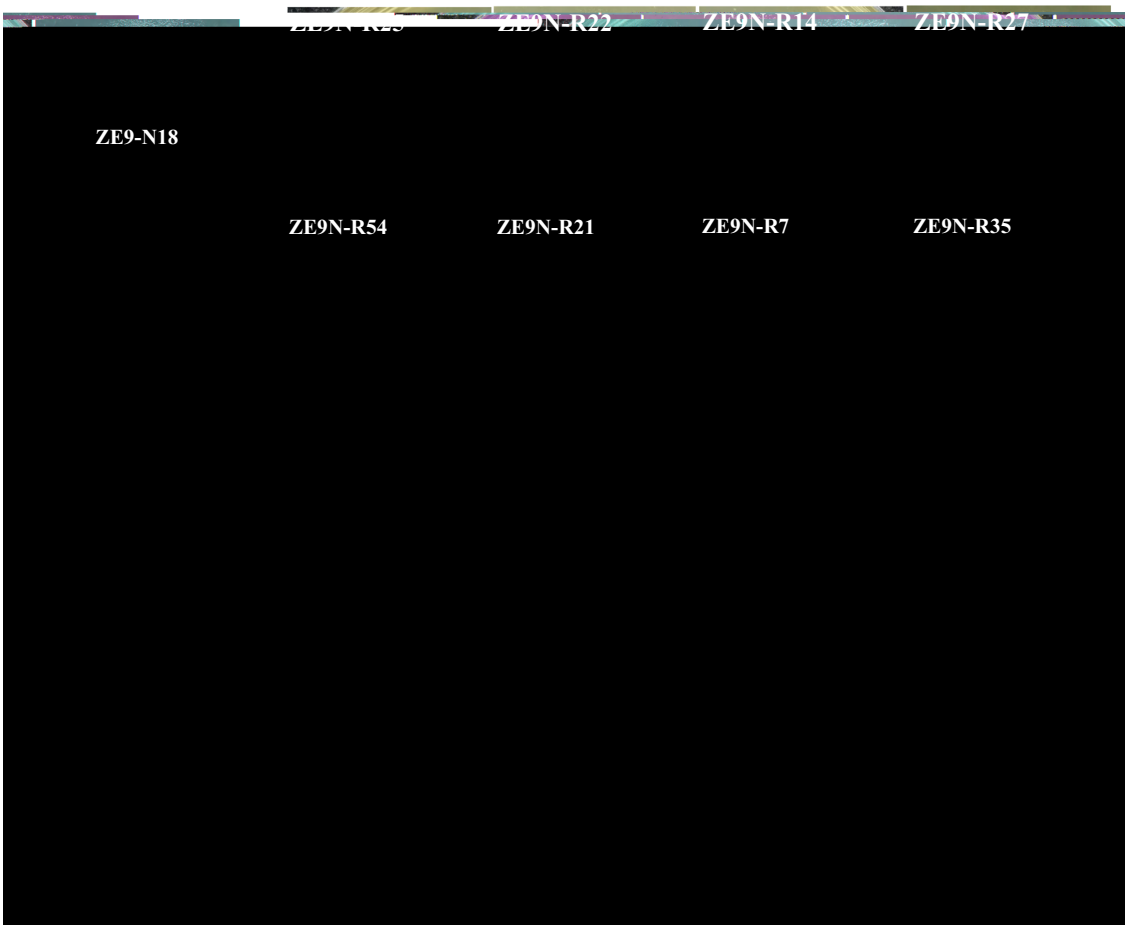


**Fig. 2** Mutant strains induced by neomycin plus rifampicin. **a** Growth of mutant colonies. **b** Detection of epothilones in mutants. The error bars represent the standard deviation of three independent experiments. The high-yielding mutants selected for further mutagenesis were marked with red asterisk

containing various mutations in the *rpsL* gene, with the exception of ZE9-P27 and ZE9-P28 (Fig. 1b, Table S1). is further demonstrates that the identified mutations do not contribute to enhance the epothilone production.

High-yielding mutants, including ZE9N-18 and the dual-resistant mutants ZE9N-R14, ZE9N-R22, ZE9N-R25, and ZE9N-R27, were analyzed by genome resequencing, which revealed only a few mutations (Table S3). In ZE9N-18, a single nucleotide polymorphism (SNP) mutation was found in *orf05968*, which encodes the CarD-like family RNAP-interacting protein CdnL [29]. is SNP resulted in the loss of the termination codon, leading to extension of the C-terminus with 32 amino acids. Notably, this alteration did not disrupt the primary function of CdnL, as it is indispensable essential in *M. xanthus*. e other SNP was found upstream of the coding region of the 50S ribosomal protein L10 (*rpsJ*), which might contribute to the acquisition of resistance to Neo. In addition, an insertion-deletion (InDel) mutation was detected in a hypothetical protein-encoding gene (Table S3). For the dual-resistant mutants, the typical mutations in *rpoB* of the four tested mutants were two novel substitutions (Q535L and H548D) not present in the ZE9-R mutant strains. In conclusion, mutant strains acquired few mutations during the RE progression, and the combined e ect of mutations resulted in changes in strain resistance, phenotype, and activated production of epothilones.

### Characteristic identi□cation of high-yielding mutants

As shown in Fig. 4a, ZE9-N18 exhibited slightly delayed exponential growth phase, accompanied by an extended stationary phase, which is theoretically conducive to the biosynthesis of secondary metabolites, such as epothilones. e combined mutations selected with Rif led to further delayed exponential growth in high-yielding ZE9N-R strains. For instance, when cultured to the midpoint of the exponential growth phase (36 h),

the biomass of ZE9-N18 was found to be 20% lower than

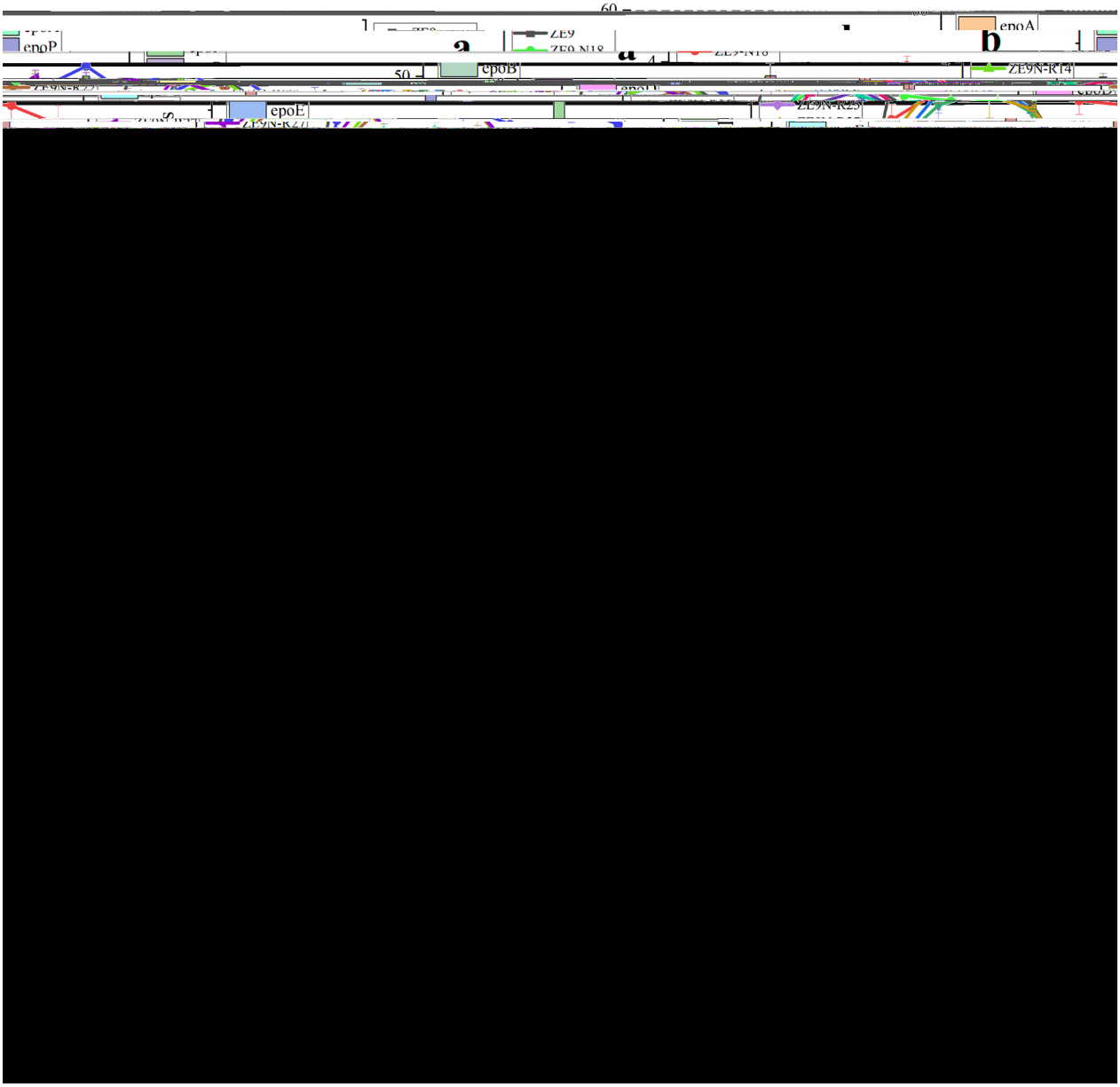


**Fig. 4** Characteristic identification of high-yielding mutants. **a** Growth curves of ZE9 and mutants. **b** Transcriptional analysis of the epothilone genes in ZE9 and di□erent mutants. **c** HPLC analysis of the secondary metabolites in ZE9 and di□erent mutants

with those in ZE9-N18, most epothilone-encoding genes were further activated in the ZE9N-R strains, especially in ZE9N-R25. While the elevated transcription of biosynthetic genes is a crucial factor contributing to epothilone production, it is not the sole determinant. Although the transcription levels of the epothilone-encoding genes in ZE9N-R25 were 1.8 to 2.5 times greater than those in ZE9N-R22 (Fig. 4b), no significant di erence in epothilone yields was detected between these two mutants. is might be attributed to the delayed growth of ZE9N-R25 compared to ZE9N-R22 (Fig. 4a). It has been indicated that mutant ribosomes may exhibit enhanced stability and facilitate the synthesis of intracellular proteins during stationary phases [1, 30]. We determined the intracellular protein content of ZE9 and its mutants; however, no significant di erences were observed (Figure S1).

At the metabolite level, the fermentation products of representative strains were analyzed with LC–MS, which revealed that, in addition to epothilones,

the synthesis of endogenous secondary metabolites was significantly a ected. Four NAPs of *M. xanthus* were identified, including three hybrid polyketide-nonribosomal peptide (PK−NRP, myxovirescin A [31], DKxanthene [32], and myxalamid [33]), as well as one ribosomally synthesized and posttranslationally modified peptide (RiPP, cittilin [34]). ese compunds consumes malonyl-CoA, methylmalonyl-CoA, and amino acids as precursors, akin to the biosynthesis of epothilones [35] (Table S4). Compared with that in ZE9, the production of myxovirescin A in the tested mutants was significantly enhanced (Fig. 4c), while the synthesis of cittilin and DKxanthene was inhibited, and the production of myxalamid remained una ected (Figure S2). e varying trends in product synthesis observed in this study further demonstrated the complex e ects of RE mutagenesis on intracellular secondary metabolism. Although no new products were identified in the high-yield mutants tested, the potential for discovering activated unknown products in other myxobacteria through RE, as demonstrated in *Streptomyces* [1], merits further investigation.

### Scaled-up fermentation of high-yielding mutant ZE9N-R22 in bioreactors

e improved epothilone titer in ZR9N-R22 was demonstrated through fermentation experiment in flasks mentioned above. To further validate its productivity, bioreactor fermentation was performed using both 3 L and 10 L fermenters. A 7 day batch fermentation of ZR9N-R22 was conducted in a 3 L bioreactor. After incubation with 10% of seed solution, the DO showed a gradual decline corresponding with the increase in bacterial growth (Fig. 5a). e pH level increased from 7.4 to 8.3 after six days of incubation in the 3 L bioreactor without any supplementation (Fig. 5b). is rise in pH might be attributed to the release of $NH_3$ during the metabolic processes of abundant amino acids in the CMO medium. e epothilone titer achieved was 29.4 mg/L, which was 46% lower than that in flasks (Fig. 5c).

A fed-batch fermentation lasting 18 days was subsequently conducted in a 10-L bioreactor. As illustrated in Fig. 5d and e**,** nitrogen nutrition and methyl oleate were added in five batches after four days of fermentation (feed nodes were indicated by red dashed lines). Each feeding e ectively promoted cell growth, resulting in an instantaneous decrease in DO abundance, while the pH remained relatively stable. After 5 feedings, the maximum biomass attained was 12.5 g/L (Fig. 5f).

e $OD_{600}$ value and dry cell weight (DCW) exhibited a decline upon the cessation of feeding. Ultimately, after 18 days of cultivation, the titers of epothilone A and epothilone B were 44.8 mg/L and 48.6 mg/L, respectively, culminating in a total yield of 93.4 mg/L (Fig. 5c). is is the highest epothilone heterologous expression yield reported in the literature [15].

## Discussion

Since the BGC of epothilone was heterologously expressed in *M. xanthus*, various genetic manipulation strategies have been applied to optimize the yield of epothilones. Based on the characterization of the promoter within the epothilone BGC [36], the original promoter was replaced or arranged in series, resulting in a 1.2-fold enhancement in epothilone production [17]. Deletion of the transcriptional inhibitor upstream the epothilone BGC led to a 4.1-fold increase in the production of epothilones [18]. Besides, the CRISPR-dCas9 system was introduced into ZE9 to activate either single [20] or multiple promoters [19] within the epothilone BGC, and promoted the epothilone synthesis by about 2 times. Compared with the above genetic manipulations, RE generates mutants exhibiting unpredictable yield of epothilones. is non-directional characteristic indicates that it may be necessary to screen a substantial number of mutants in order to identify high-yielding strains. However, RE is independent of the genetic flexibility of the strain, which makes the method particularly advantageous for application in non-model bacteria.

Although RE has been demonstrated to be e ective in activating the synthesis of secondary metabolites, the mechanism of such activation is still unclear. A mutation in *rpsL* was identified in Str-resistant *Streptomyces* mutants with activated production of NAPs [1], which has been explained by the maintained translational accuracy [37] or increased stability of ribosomes [3]. Certain *rpsL* mutations reportedly increase protein synthesis during later stages of growth, which coincides with NAP production in most microorganisms [38]. However, some studies have refuted the causal relationship between simple mutations in ribosomal proteins and increased product yield. For example, the direct introduction of mutations in *rpsL* or *rsmG* resulted in resistance to Str, but did not improve the production of tiancimycins [39]. In our study, mutants harboring the same mutation exhibited di erent epothilone production. is also demonstrates the intricate nature of the activation on the production of NAPs by RE.

Mutations in *rpoB* during the Rif selection have been shown to alter the a nity of RNAP for specific promoters, e ectively recognizing and transcribing silenced BGCs and thereby promoting product synthesis [1]. e mutations confering resistance to Rif have been well reviewed [40]. Most (>95%) of the mutations are clustered within four distinct sites within the *rpoB*

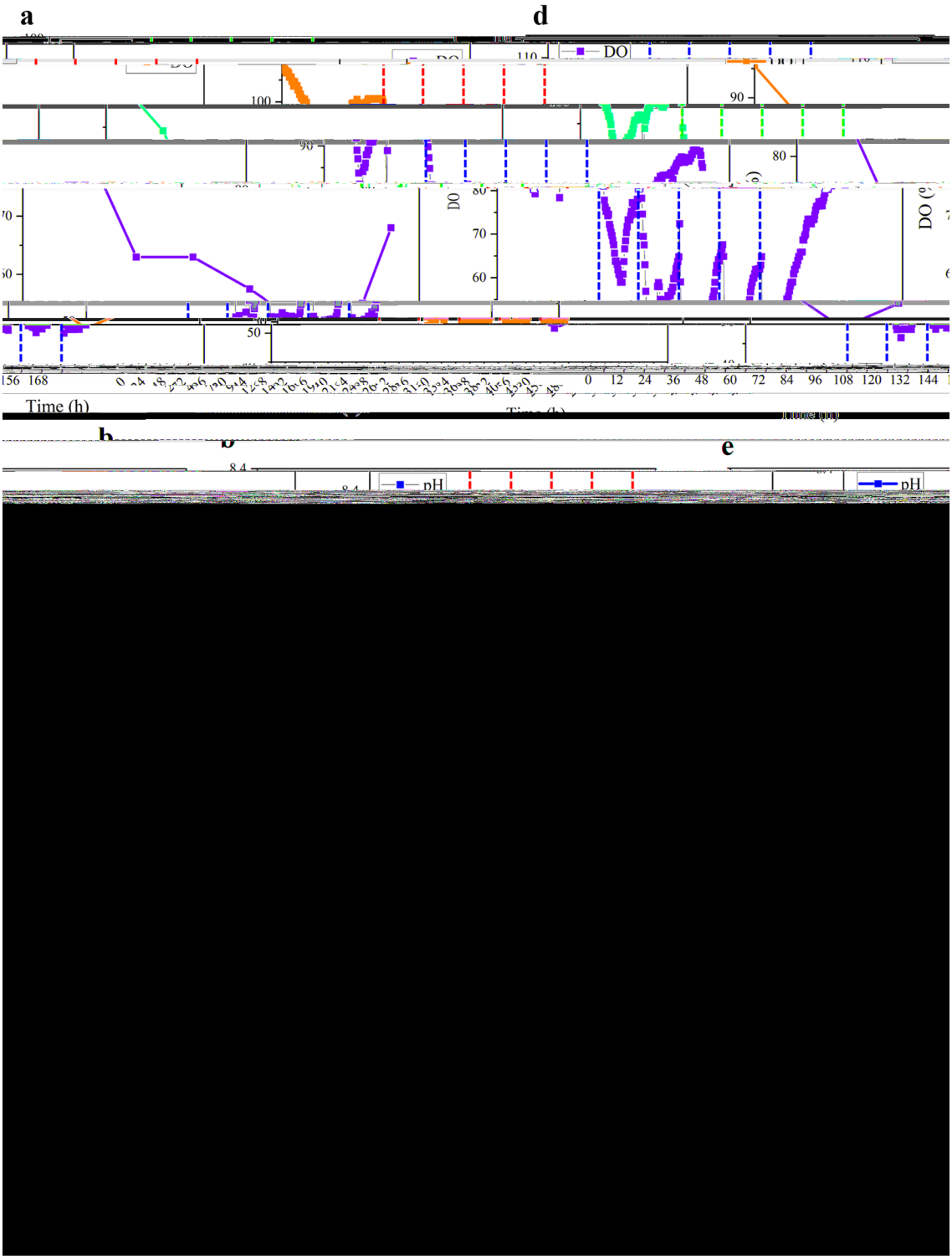


**Fig. 5** Fermentation of ZE9N-R22 in bioreactors. **a** DO curve in 3 L fermenter. **b** pH curve in 3 L fermenter. **c** Yields of epothilones in flasks and fermenters. **d** DO curve in 10 L fermenter. **e** pH curve in 10 L fermenter. **f** Biomass detection in 10 L fermenter. The red dotted line indicated the feeding time nodes

gene, the so-called Rif cluster N, I, II and III (Figure S3). We compared the Rif cluster in *M. xanthus* with the sequences summarized in the literature [40, 41], and marked the mutations identified in this study (Red mark in Figure S3). No novel mutation was found in the *M. xanthus* mutants we obtained. Although certain mutations, such as D427V, S433P, S433L, H437Y, H437R, have been identified as particularly e ective in activating the production of actinorhodin and undecylprodigiosin in *Streptomyces*, their applicability may not extend to other organisms. Apparently, enhanced production of epothilone was not observed in the ZE9-R1/ZE9-R2 strain containing the D538V mutation (corresponding to the D427V in *Streptomyces*), nor in the ZE9-R3/ZE9-R4 harboring the H548Y (corresponding to the H437Y in *Streptomyces*).

Among the mutants derived from RE that exhibit enhanced production of NAPs, certain strains demonstrated significant alterations in growth characteristics, such as the delayed sporulation in *Streptomyces diastatochromogenes* [42] and slender

**Abbreviations**

| | |
|---|---|
| RE | Ribosome engineering |
| Rif | Rifamycin |
| Str | Streptomycin |
| Par | Paromomycin |
| Gen | Gentamicin |
| Neo | Neomycin |
| Tet | Tetracycline |
| Spe | Spectinomycin |
| NAPs | Natural active products |
| DO | Dissolved oxygen |
| DCW | Dry cell weight |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02627-3.

Supplementary Material 1.

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**Author contributions**
XJY, XK, and YZL designed the researches; XK, XRY and CXW performed researches; XJY, XK, and XRY analyzed the data; XJY wrote the main manuscript text; JRW, JNZ, ZPY, QKF, WH, CSW and YZL revised the manuscript. All authors reviewed the manuscript.

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**Data availability**
Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the primary accession code PRJNA1184456.

## Declarations

**Competing interests**
The authors declare no competing interests.

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